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RESEARCH ARTICLE

Aureobasidium pullulans as a biocontrol agent of postharvest pathogens of apples in Uruguay

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Aureobasidium pullulans was the microorganism most frequently recovered from the surface of apple fruit (cv. Red Delicious) stored in commercial cold chambers for 6 months. In the present work, 10 isolates of *Aureobasidium pullulans* were assayed to determine if they could control blue and grey mold disease of apple during cold storage. Although nine of 10 isolates, significantly reduced the percentage of decayed wounds when compared to the control, one of them, designated isolate ApB, showed the highest levels of protection. ApB was able to grow in a wide range of temperatures lower than 35°C, which is an important human health safety factor. ApB was resistant to thiabendazole, iprodione and imazalil, the most commonly commercially applied fungicides in postharvest treatment of apples in Uruguay. Regarding the mechanisms of action of the selected biocontrol agent, lytic enzymes did not seem to play a central role. ApB depleted iron from nutrient media, which may be an important aspect of its ability to inhibit *Botrytis cinerea*. Further experiments are needed, however, to determine if the depletion of iron is caused by the production of siderophores, by the immobilization of iron in an insoluble pigment, or a combination of both.

Keywords: biocontrol; apples; postharvest; *Aureobasidium pullulans*

Introduction

Depending on the commodity and available technologies, losses of fruits and vegetables during postharvest storage can be high. In Uruguay, Falchi (2001) stated that fungal pathogens are the main cause of those losses with *Penicillium expansum* Link and *Botrytis cinerea* Pers.:Fr being the most commonly responsible. In Uruguayan packing houses, apples which are going to be stored less than 3 months do not receive any fungicidal treatment after harvest, but if the storage period is longer, postharvest applications of thiabendazole, iprodione or imazalil are commonly used. However, fungicide efficacy is frequently decreased by the presence of resistant strains of a pathogen. Moreover, public concern about the use of chemical pesticides, especially at the postharvest stage, has increased. Therefore, new strategies to reduce or replace fungicides have been proposed.

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The use of antagonistic microorganisms as biocontrol agents to inhibit postharvest pathogens has shown great potential (Droby, Wisniewski, Macarasin, and Wilson 2009). Many yeast species have been reported as good antagonists against postharvest diseases in fruit including the yeast-like fungus *Aureobasidium pullulans* (deBary) G. Arnaud. *A. pullulans* is an effective biocontrol agent of postharvest diseases on various kinds of fruit including apple (Castoria et al. 2001; Schena, Nigro, Pentimone, Ligorio, and Ippolito 2003). Production of antifungal enzymes (chitinases and glucanases), nutrient competition and host defense induction are possible modes of action for strains of this species (Ippolito, El Ghaouth, Wilson, and Wisniewski 2000; Castoria et al. 2001; Bencheqroun et al. 2007). However, the majority of studies on *A. pullulans* have been performed at 25°C, not at the postharvest temperature at which fruit is stored. Moreover, nutrient competition studies of this antagonist have been mainly focused on competition for nitrogen (Bencheqroun et al. 2007). To our knowledge, studies on competition for micronutrients such as iron have not been conducted.

A. pullulans is an ubiquitous and widespread oligotroph that can be found in environments with fluctuating water activities. Typical temperature range for growth is 2–35°C. However, strains of this species have been recovered from very cold environments, like Artic ice (Zalar et al. 2008), and there are also references of human pathogenic isolates with a higher maximum growth temperature (Samson, Hoekstra, Frisvad, and Filtenborg 1995). These data highlight the importance of determining the temperature range for growth of a potential biocontrol agent during the selection process, especially if the antagonist is to be applied on food.

Despite considerable research in this area and efforts at commercialization of biocontrol products, their widespread use as a postharvest treatment has not been fully realized (El-Ghaouth, Smilanick, and Wilson 2000a). In an effort to enhance the reliability and efficacy of postharvest biocontrol agents, studies have been conducted combining microbial antagonists with additives that could improve their performance. Low doses of fungicides and chitosan are among the tested additives (El-Ghaouth et al. 2000a; Yu, Li, and Zheng 2007). Since both of these additives exhibit antifungal action themselves, their use in combination with microbial antagonists needs to be tested to ensure that the combination is compatible and that the biocontrol agent is not detrimentally affected. Other additives have also been studied including, sodium bicarbonate, calcium chloride, nitrogenous compounds, and the sugar analogue 2-deoxy-D-glucose (El-Ghaouth, Smilanick, Wisniewski, and Wilson 2000b; Nunes, Usall, Teixidó, Miró, and Viñas 2001; Porat, Daus, Weiss, Cohen, and Droby 2002; Yao, Tian, and Wang 2004). In each case it is important to understand the mechanisms involved in biocontrol in order to select the right additive.

In the present study, 10 isolates of *A. pullulans* were evaluated as biocontrol agents against blue and grey mold in apples, caused by *P. expansum* and *B. cinerea*, respectively. The isolate which gave the highest protection was selected and its identity confirmed by DNA sequence analysis of the ITS1–ITS2 region. We also determined that the selected strain dies at 37°C which is important as a human health safety factor. Sensitivity of the isolate to fungicides was determined *in vitro* and on fruit and the possibility of combining the isolate with chitosan to potentiate the biocontrol activity was also evaluated. Furthermore, we demonstrated that the selected isolate produced

chitinases and glucanases at low temperatures, in the presence of pathogen cell walls, and that hydroxamate-type siderophores may be involved in biocontrol.

Materials and methods

Fruit

Apples (*Malus* × *domestica* Borkh cultivar 'Red Delicious') of uniform size, without wounds or rot, and with no postharvest treatment were used in this study. For isolation of biocontrol microorganisms, apples that had been stored for 6 months in five different commercial storage chambers were used. For biocontrol assays, apples were used within 1 week.

Pathogens

Strains of *P. expansum* and *B. cinerea*, selected for their high level of aggressiveness on Red Delicious apples (Pianzzola, Moscatelli, and Vero 2004) were used in this study. Cultures were maintained on PDA at 5°C.

Isolation and selection of biocontrol agents

Potential biocontrol agents were selected from cultures of the superficial microflora present on apples stored for 6 months at 0–1°C. Apples from 10 different orchards, stored in five different commercial cold chambers were used. Three fruits from different locations were aseptically peeled and the peels were suspended in 300 mL of sterile water and homogenized in a Stomacher for 3 min. Serial dilutions of these homogenates were plated on Apple Juice Agar (Apple Juice and 2% Agar, pH 4.5) and incubated at 5°C for 10 days. Microorganisms recovered were identified to different taxonomic levels. Bacteria were identified to family level based on Bergey's manual identification keys (Brenner 1984). Further identification was performed using Enterotube™ II from BBL™, Becton Dickinson, Inc. Yeasts were identified to genus and species level according to Kurtzman and Fell (1998).

Biocontrol assays

Ten isolates of the most frequently isolated yeast species were evaluated as biocontrol agents against *P. expansum* and *B. cinerea* in apple wounds at 1°C.

Apples within 1 week of harvest were surface disinfected with sodium hypochlorite (0.1%) for 2 min and then rinsed with running tap water. Five wounds (3 × 2 mm, deep × wide) were made along the equator of each apple with a cork borer.

Four wounds were inoculated with 10 μL of a microbial suspension (10^7 cfu/mL) of the biocontrol agent and the remaining wound with 10 μL of sterile water as a control. Antagonists suspensions were prepared in 5 mL of sterile water, using 2-day-old potato-dextrose agar (PDA) cultures to reach a concentration of 10^7cfu/mL After 2 h, wounds were inoculated with 10 μL of a conidial suspension (10^4 conidia/mL) of either pathogen. This pathogen concentration had been previously shown to produce 100% infection of wounds (Vero, Mondino, Burgueño, Soubes, and Wisniewski 2002). Forty

replicates per treatment were performed. Two parameters were recorded: percentage incidence (infection rate) and percentage severity as (lesion diameter in treated wounds/lesion diameter in control wounds) $\times 100$. Experiments were repeated twice.

The isolate that exhibited the highest level of disease control against both pathogens was selected for further study. With the selected antagonist a larger, confirmatory biocontrol assay was performed as described above where $n = 80$. Experiments were repeated twice.

Biocontrol agent identification

The identity of the selected antagonist as *Aureobasidium pullulans* isolate B (ApB) was confirmed by sequencing the ITS1-5.8rDNA-ITS2 region. DNA was extracted as described by Schena et al. (1999). PCR fragments were generated using primers ITS 4 and ITS 5 (BYO.SYNTHESIS Company, Lewisvile, TX, USA) covering the ITS 1, 5.8S and ITS 2 region of ribosomal DNA (White, Bruns, Lee, and Taylor 1990). Ready-to-Go PCR Beads (Amersham Pharmacia Biotech, Piscataway, NJ, USA) were used for amplification reactions. Amplifications were performed with an initial denaturation step for 2 min at 94°C, followed by 35 cycles, with a final extension of 10 min at 72°C. Each cycle consisted of the following steps: denaturation (40 s at 95°C), annealing (1 min at 54°C), and extension (2 min at 72°C). PCR was performed with a Perkin-Elmer, Model 2400 thermocycler (Waltham, MA, USA). Sequencing of the purified PCR product was performed at Macrogen Inc. (Seoul, South Korea). Sequence similarity searches were performed with BLAST network service of the NCBI database http://blast.ncbi.nlm.nih.gov/Blast.cgi.

Biocontrol agent temperature dependent growth rate

Growth curves of ApB were constructed by constructing a temporal profile of the log of viable cell concentration from cultures of ApB in sterilized apple juice at different temperatures (5, 10, 25, 30 and 37°C). For each growth curve, growth rate was determined as the slope of the exponential phase. Three replicates of each growth curve were performed. Growth rates were plotted against temperature and optimum growth temperature was determined as the temperature at which growth rate was the highest.

Sensitivity of ApB to fungicides and chitosan

Sensitivity of ApB to several fungicides was assessed. Minimal inhibitory concentration (MIC) of iprodione, thiabendazol and imazalil was determined in PDA amended with different fungicide concentrations. Inoculum of ApB was prepared as described before. The suspension was adjusted to 10^4 cfu/mL and 10 μL of suspension was inoculated onto 200 μL of PDA amended with different fungicide concentrations dispensed into the wells of sterile, disposable, 96-well microtiter plates (Montegrotto Terme, Padova, Italy). After 72 h of incubation at 25°C in darkness, microbial growth was determined visually. The minimal inhibitory concentration was defined as the lowest concentration that inhibited microbial growth. Fungicide concentrations assayed were 0, 1, 2, 4, 8, 16, 32, 64, 128, 256 and 512 μg/mL. Formulated fungicides were used for these experiments. Rovral 50 WP (Rhône Poulenc, Lyon, France), Tecto 500SC (500 g/L; Syngenta International AG, Basel, Switzerland) and Fungaflor

500EC (Janssen Pharmaceutica N.V., Bélgica) were used for iprodione, thianbendazole and imazalil, respectivley. The amount of formulated fungicide added to the medium was calculated to reach the concentrations of active ingredients specified above. The experiment was repeated three times.

MIC of chitosan was also determined for ApB in apple juice. Spores of ApB (10 μL of a suspension containing 1×10^4 cfu/mL in sterile distilled water) were transferred to 10 mL tubes containing 5 mL of sterile apple juice amended with 0, 0.05, 0.1, 0.2, 0.5, 1 and 2% chitosan chloride. Chitosan chloride solutions were prepared as described by Rey Barrera (1998). Chitosan used in this work was $\geq 85\%$ deacetylated (Sigma Chemical Co., St Louis, MO, USA). Three replicates were performed for each chitosan chloride concentration.

Effect of chitosan on biocontrol

Chitosan chloride (1%) and a combination of ApB with 1% chitosan chloride were evaluated for suppression of blue mold development. Biocontrol assays were performed as described above, but in this case, apple wounds were inoculated with 10 μL of 1% chitosan chloride alone and in combination with ApB at a concentration of 10^7 cfu/mL prior to pathogen inoculation. Each experiment consisted of 80 replicates and experiments were repeated twice.

Wound colonization by ApB in the presence or absence of chitosan chloride

Growth curves of ApB in apples wounds were performed at 1°C. The effect of chitosan chloride on survival of ApB in apple wounds was also determined. Apples were wounded as described previously but with only one wound per apple. Wounds were inoculated with 10 μL of a yeast cell suspension (1.0×10^7 cfu/mL) in either sterile water or 1% of chitosan chloride. After inoculations, fruit were placed in boxes at 1°C. At each collection time (3, 12, 43, 63 and 76 days), the surrounding tissue from the wound of three different apples was removed and placed in a 1.5 mL Eppendorf tube containing 1 mL of aqueous sterile solution of Tween 20 (0.1%). Tubes were homogenized in a vortexer for 2 min. Quantification of ApB viable cells in each sample was performed by plate count on PDA. Experiments were repeated three times.

Wound colonization by ApB in the presence of fungicides

The ability of ApB to grow in apple wounds in the presence of imazalil was assessed. The assay was performed in parallel with the one described above. In this case, each wound was inoculated with ApB in sterile distilled water as previously described. After 2 h at room temperature, inoculated fruit was submerged in either water (control) or a fungicide suspension at a commercial use concentration (500 μg/mL) and half of that concentration (250 μg/mL) for 30 s. Fruit was then, dried at room temperature for 2 h before cold storage at 0–1°C. Quantification of ApB viable spores in wounds at different times (1, 5, 10, 15, 23 and 30 days) was evaluated as described above. Experiments were repeated three times.

Mechanisms of biocontrol

In vitro *antagonism*

The ability of ApB to inhibit pathogen growth was tested on dual cultures on apple juice agar using both pathogens as described by Spadaro, Vola, Piano, and Gullino (2002). Briefly, a line of the antagonist was plated on the medium from a single drop of a spore suspension. A total of 90-mm Petri dishes were used and the strip of antagonist was approximately 25 mm from the border. A 5-mm mycelial disk of the pathogen was placed 30 mm from the border and 30 mm from the antagonist. Plates were incubated at 25°C until the mycelium of the pathogen reached the Petri dish border opposite the antagonist strip. The radial growth of the pathogen towards the antagonist was then measured and was compared to the opposite colony radius (30 mm). Experiments were repeated twice.

Competition for iron

Dual cultures of ApB with native strains of *P. expansum* and *Botrytis cinerea* in the presence of low concentrations of iron (0, 10, 100 and 500 μM of ferric chloride) were plated. The medium used in this assay was the same as described by Calvente, Benuzzi, and Sanz de Tosetti (1999). Production and chemical nature of siderophores was also assessed as described by Calvente, de Orellano, Sansone, Benuzzi, and Sanz de Tosetti (2001). Briefly, the protocol involves inoculating ApB on agar plates containing a medium designed for siderophore production (sucrose 25 g/L, ammonium sulphate 4 g/L, potassium dibasic phosphate 3 g/L, citric acid 1 g/L, magnesium sulphate 0.08 g/L, zinc sulphate 0.002g/L and agar 20 g/L). After 48 h of incubation at 25°C the detection of siderophores was evidenced by the formation of orange reddish halos around colonies after applying a ferric perchlorate solution (20 mM ferric chloride − 0.1 M perchloric acid) to the plates. The appearance of colored halos indicated the presence of hydroxamate-type siderophores.

Effect of ApB of pathogen spore germination

The effect of ApB antagonist on spore germination of *P. expansum* and *B. cinerea* was assessed in sterile apple juice as described by Spadaro et al. (2002). Aliquots (100 μL) of spore suspension (1×10^7 spores/mL) of each pathogen in sterile distilled water were transferred to 10 mL tubes containing 5 mL of sterile apple juice. Living spores of ApB (100 μL of a suspension containing 1×10^7 spores/mL) were added to each tube, except for control tubes. After 12 h incubation at 25°C, 100 spores per replicate were observed microscopically and their germination was evaluated. The treatments were repeated three times.

Production of antifungal enzymes

Production of chitinase and β-1,3-glucanase by ApB was assayed in the presence of fungal cell walls (FCW). FCW were prepared as described by Rey Barrera (1998) with slight modifications. *P. expansum* native strain was grown on Yeast Extact Sucrose medium (YES) at 25°C for 5 days. Obtained mycelia were dried with sterile

filter paper and ground in a sterile mortar in the presence of liquid nitrogen to get a fine powder. The mycelial powder was suspended in 5M NaCl, sonicated for 5 min and centrifuged at 5724 $\times g$ for 20 min. The supernatant was discarded and the pellet was washed three times with distilled water. FCW were dried in Petri dishes at 60°C for 3 h. Dried FCW were added to Yeast Nitrogen Base (YNB) or Apple Juice to reach a concentration of 1 mg/mL and then autoclaved. The resultant media were inoculated with ApB and incubated at 5°C for 3 weeks. In parallel, apple juice without FCW was also inoculated as described above. At different times (3, 7, 20, 25, 28 and 30 days), aliquots from the three different cultures were filtered through a 0.45-μm filter. Enzymatic activities were assayed in the filtrates previously purified through a Sephadex G-25 column (PD-10; GE Healthcare, Uppsala, Sweden) equilibrated with 0.05 M potassium acetate buffer (pH 6), to remove monosaccharides. At each sampling period the concentration of viable spores of ApB was determined by plate count on PDA at 25°C.

Chitinase activity was assayed as described by Mahadevan and Crawford (1997) by measuring the release of *p*-nitrophenol from *p*-nitrophenyl *N*-acetylglucosamidine [pNP(GlcNAc)]. The pNP(GlcNAc) (Sigma Chemical Co.) was dissolved in 0.05 M potassium acetate buffer (pH 6). Culture filtrate (10 μL) was added to 90 μL of 0.18 mmol/L *p*-nitrophenyl reagent in a microtiter plate and incubated at 37°C for 6 h. The reaction was stopped by adding 10 μL of 1 mol/L NaOH. Absorption was measured at 405 nm in a Microplate Autoreader (Bio-tek Instruments, Winooski, VT, USA). One unit of enzyme was defined as the amount releasing 1 μmol of paranitrophenol per mg protein.

β-1,3-Glucanase was assayed as in Masih and Paul (2002). A reaction mixture was prepared by adding 62.5 μL of 0.05 M potassium acetate buffer (pH 6) containing 1% laminarin to 62.5 μL of culture filtrate and incubated at 37°C for 6 h. Glucanase activity was determined as the amount of reducing sugars released from laminarin, measured according to the Nelson–Somogyi method (Nelson 1944; Somogyi 1952) using glucose as a standard. One unit of β-1,3-glucanase was defined as the amount of enzyme releasing 1 μmol of reducing sugars mg of protein^{-1}.

Protein content was measured as described by Bradford (1976) with bovine serum albumin (Sigma A-9647) as a standard.

The optimal temperature of enzymatic activity for the crude chitinase and glucanase preparations was also determined. Temperatures assayed were 1, 5, 25, 30, 37 and 45°C. All experiments were repeated three times.

Statistical analysis

The data on disease severity were subjected to analysis of variance (ANOVA). Differences between means were tested using least significant difference. Disease incidence was analyzed with the same model but a binomial distribution and logit transformation were used (maximum likelihood). Contrasts between means were performed when significant effects were found (Toutenburg 1995). All analysis were performed in Statistical Analysis System, Release 6.12 (SAS/STAT®, 1996, SAS Institute, Cary, NC, USA) PROC GENMOD.

Results

Isolation, selection and identification of biocontrol agents

Approximately 10^6 CFU/apple of superficial microorganisms were recovered using the assay conditions in this study. Forty individual isolates were recovered which included both fungi and bacteria. Fungi were found in higher numbers in all samples. *Pantoea agglomerans* was the predominant bacterial species while *Aureobasidium pullulans* was the predominant fungal species. *Rhodotorula* spp. and *Cryptococcus* spp. were also found but in lower numbers. Ten different *Aureobasidium pullulans* isolates were recovered and evaluated as biocontrol agents against blue and grey mold in apple.

In the first biocontrol assay, nine of the 10 tested isolates significantly reduced the percentage of decayed wounds when compared to the control (Figure 1). However, ApB, showed the highest biocontrol activity against both pathogens.

Protection levels achieved in a second biocontrol assay with ApB (Figure 2) using a larger number of fruits were nearly the same as obtained in the first assay (72 and 80% protection for blue and grey mold, respectively). ApB was then selected for further study. Identification of isolate B as *Aureobasidium pullulans* was confirmed by the evaluation of the obtained ITS1–ITS2 sequence. Comparison of this sequence with the NCBI data base indicated a 100% homology with sequences corresponding to *Aureobasidium pullulans*.

Temperature dependent growth rate and sensitivity to chemical fungicides

Optimal growth temperature of ApB was 25°C and no growth occurred at temperatures over 35°C. At temperatures higher than 35°C, growth rates had negative values, indicating that these temperatures were injurious or lethal (Figure 3).

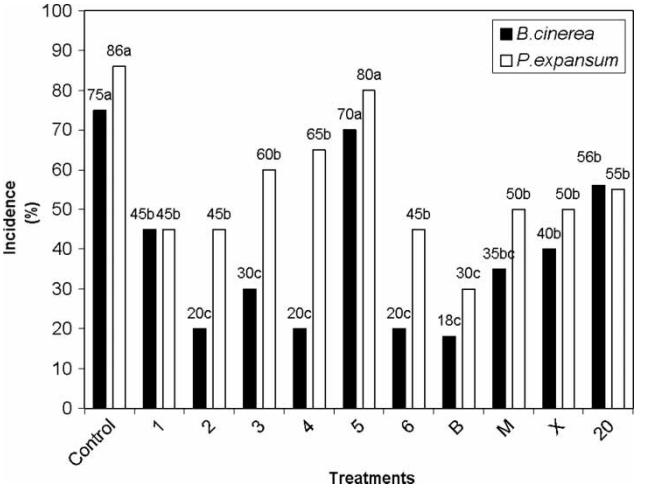

Figure 1. Incidence of blue and grey mold on apples inoculated with different *Aureobasidium pullulans* strains and *Penicillium expansum* or *Botrytis cinerea*. Controls were not treated with antagonists. Fruit were held for 3 months at 0–1°C. Treatments with the same letter are not significantly different ($P = 0.05$).

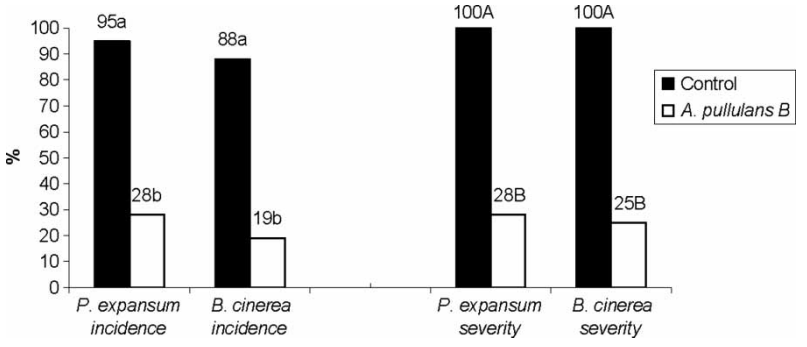

Figure 2. Incidence and severity of blue and grey mold on apples inoculated with *Aureobasidium pullulans* strain B and *Penicillium expansum* or *Botrytis cinerea*. Controls were not treated with antagonists. Fruit were held for 3 months at 0–1°C. Treatments with the same letter are not significantly different ($P = 0.05$).

ApB was resistant to 512 μg/mL of iprodione and thiabendazole *in vitro,* however, the MIC of imazalil was 2 μg/mL. Growth of ApB in fruit wounds was also affected by commercial doses of imazalil (Figure 4). Growth in apples treated with 250 μg/mL of imazalil was not significantly different from the control but treatment with a commercial dose (500 μg/mL) of imazalil significantly inhibited the growth of ApB in apple wounds during the first month of cold storage. After that, the yeast population increased to nearly the same level observed in control wounds.

Chitosan chloride effect on in vitro *growth, wound colonization and biocontrol activity*

Chitosan chloride at concentrations equal or higher than 2% inhibited *in vitro* growth of ApB. In apple wounds, the population of ApB decreased in the absence of chitosan chloride more than 10-fold in the first 24 h following wound inoculation, followed by a slight increase during the incubation period (Figure 5). In contrast, in

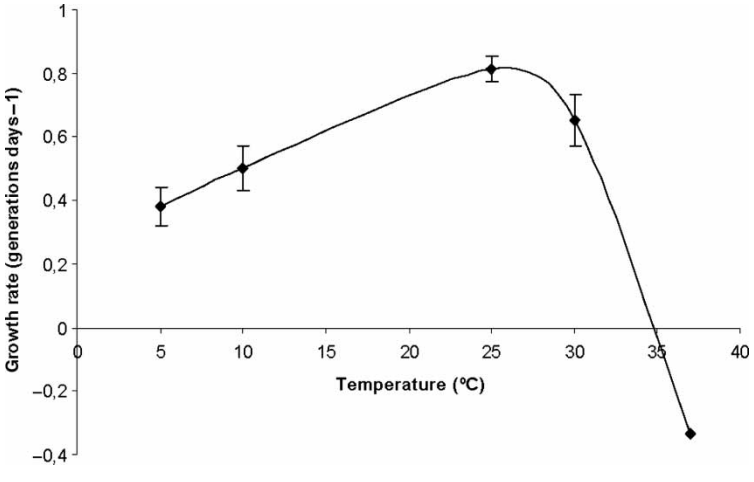

Figure 3. Effect of temperature on growth rate of *Aureobasidium pullulans* strain B in apple juice. Bars represent standard deviations.

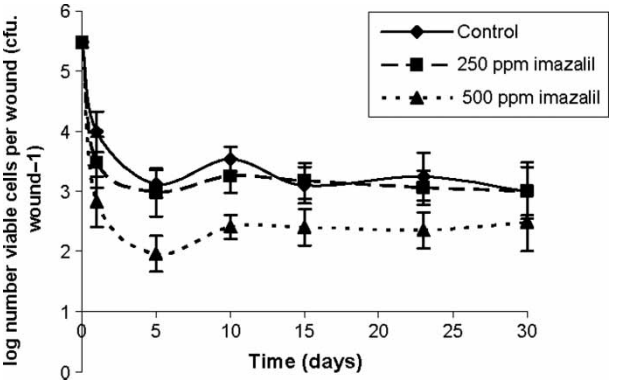

Figure 4. Growth curves of *A. pullulans* isolate B in apple wounds at 1°C, after treatment with water (Control), 250 μg/mL imazalil and 500 μg/mL imazalil. Bars represent standard deviations.

the presence of chitosan chloride, the initial ApB population decreased almost 1000-fold in the first 24 h. However, inhibition decreased as the incubation time increased. After 80 days at 1°C, the population of ApB in presence of chitosan was only 10-fold lower than in the absence of it.

Both chitosan chloride and ApB significantly reduced blue mold incidence in treated wounds (Figure 6). However, the combination of ApB and chitosan chloride together in the same wound did not result in any additional control of blue mold compared to each treatment alone.

Mechanisms of biocontrol

Several physiological and biochemical characteristics of ApB that potentially could be associated with its biocontrol activity were studied.

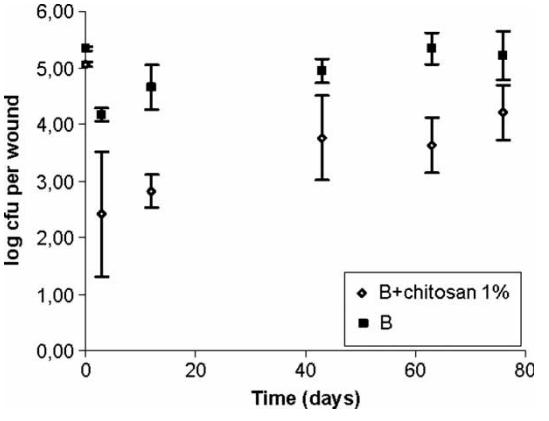

Figure 5. Growth curves of *A. pullulans* isolate B in apple wounds at 1°C, in the presence and in absence of 1% chitosan chloride. Bars represent standard deviations.

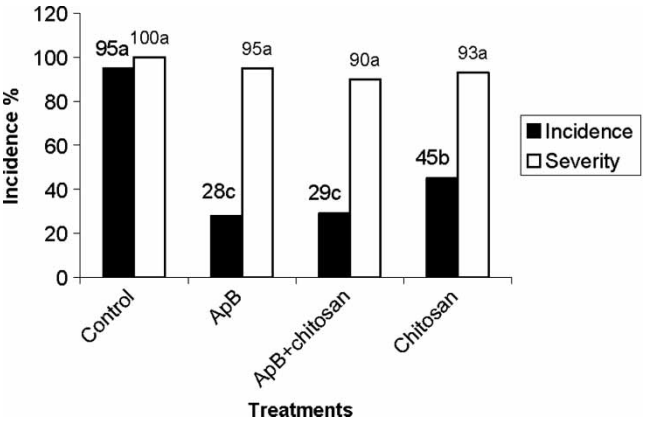

Figure 6. Incidence and severity of blue mold on apples treated with *Aureobasidium pullulans* strain B, chitosan chloride, and both, previous to the inoculation with *Penicillium expansum*. Controls were inoculated only with *Penicillium expansum*. Fruit were held for 3 months at 0–1°C. Treatments with the same letter are not significantly different ($P = 0.05$).

In vitro *antagonism and competition for iron*

A 16% inhibition of *Botrytis cinerea* radial growth was observed when dual cultures were grown on apple juice agar (data not shown). Evidence of competition for iron was observed when dual cultures were grown on media amended with low levels of iron (Figure 7). The level of inhibition of radial growth of *B. cinerea* was greater (50%) in the absence of iron; however, inhibition was lost in media containing 100 μM ferric chloride or greater (Table 1). The appearance of an orange pigment under and near *A. pullulans* colonies was observed at the higher concentrations of iron. The assay performed to detect hydroxamate-type siderophore production by ApB was positive as indicated by the appearance of reddish orange halos around colonies after the application of ferric perchlorate on assay plates (Calvente et al. 2001). In contrast to *B. cinerea,* inhibition of *P. expansum* was not observed on apple juice agar or in medium with low iron concentrations.

Effect of ApB on pathogen spore germination

Spore germination of both pathogens in apple juice was significantly reduced in the presence of living cells of ApB. An inhibition of 75 and 78% was determined in case of *P. expansum* and *B. cinerea*, respectively.

Production of antifungal enzymes

Glucanase and chitinase production was assessed in the presence of fungal cell walls at low temperatures (5°C). Glucanase activity reached a maximum (168 U mg protein^{-1}) after 25 days of growth in YNB with fungal cell walls as the only carbon source. In apple juice, activity was also maximal at day 25 but was slightly lower than in YNB (161 U mg protein^{-1}). A lag phase of about 7 days was assessed for glucanase production in both media. Maximal production of chitinase, under the same conditions was observed after 24 days in apple juice and 30 days in YNB. In

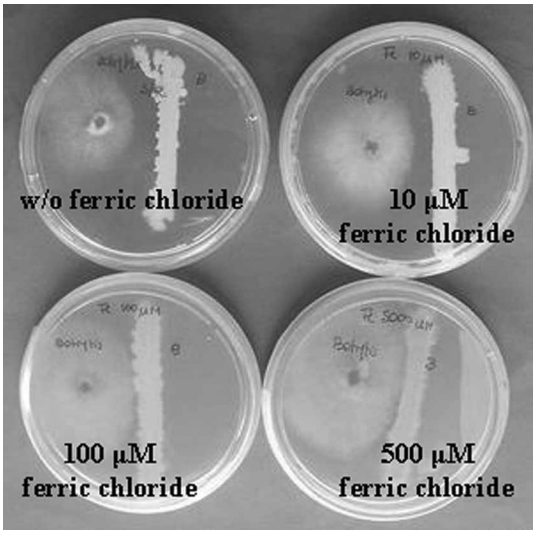

Figure 7. Dual cultures *of Aureobasidium pullulans* strain B and *Botrytis cinerea* in media with different concentrations of ferric chloride.

apple juice without fungal cell walls, maximal chitinase activity was 10 times lower than with cell walls present (Figure 8).

Growth of ApB in media amended with powdered mycelia obtained from *P. expansum* was different in apple juice than it was in YNB (Figure 9). In apple juice, ApB exhibited diauxic growth characterized by a first period with high growth rate followed by a slower growth rate which began at the same time that chitinase production increased. No lag phase was observed when ApB was grown in apple juice. In YNB, however, a lag phase of 7 days was observed before the onset of rapid growth. After 19 days, however, cell density was nearly the same in both YNB and apple juice (Figure 9). Viable cells in both media could only be detected up until the 19th day of growth. After that, ApB attachment to fungal cell wall particles, forming agglomerates, became very evident resulting in a non-homogeneous distribution of ApB in the culture medium (data not shown). Optimal enzymatic activity occurred at 45°C for both chitinase and glucanase. At 5°C activity for chitinase and glucanase resulted in 16 and 57% of the optimal value, respectively.

Table 1. Inhibition of *B. cinerea* radial growth in dual cultures with ApB in presence of different concentrations of iron.

Ferric chloride concentration (ppm)	Inhibition of *B. cinerea* growth (%)
0	50
10	27
100	5
500	0

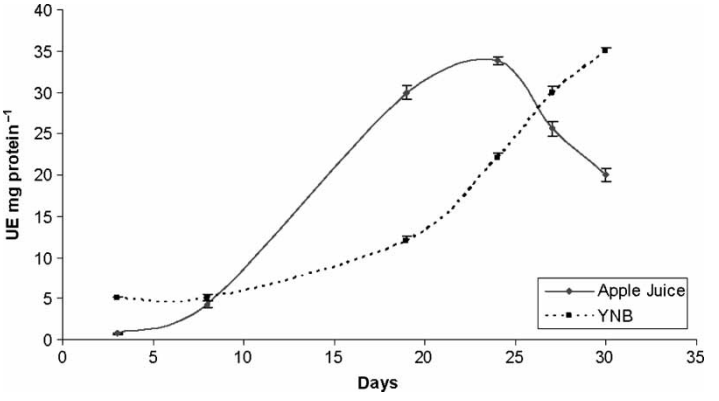

Figure 8. Effect of culture media on chitinase production by *Aureobasidium pullulans* strain B in the presence of *Penicillium expansum* cell walls. Bars represent standard deviations.

Discussion

A. pullulans was the microorganism most frequently recovered from the surface of apple fruit (cv. Red Delicious) stored in commercial cold chambers for 6 months, regardless of the orchard or the cold chamber where the fruit were obtained. This clearly demonstrates the capacity of isolates of this species to survive and colonize apple fruit peel during cold storage at 1°C. *A. pullulans* has been previously recognized as a good biocontrol agent for postharvest diseases of apples, grapes, cherries and strawberries (Lima, Ippolito, Nigro, and Salerno 1997; Schena et al. 1999; Ippolito et al. 2000; Castoria et al. 2001). Variability in the biocontrol capacity of different strains of the same species has been demonstrated (Schena et al. 2003). In the present study, although nine of the 10 isolates of *A. pullulans* tested, significantly reduced decay when compared to an untreated control, ApB consistently showed the highest levels of protection.

ApB had high levels of resistance to thiabendazole and iprodione, two of the most common commercially applied fungicides in the postharvest treatment of

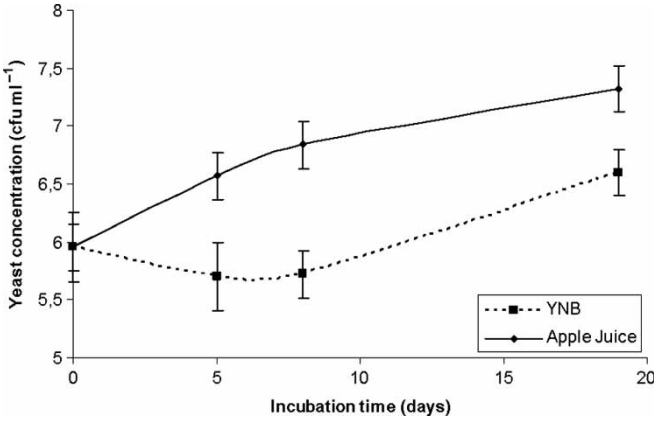

Figure 9. Growth curves of *Aureobasidium pullulans* strain B in different media amended with *Penicillium expansum* cell walls. Bars represent standard deviations.

apples in Uruguay. This implies that isolate B could be applied to fruit in combination with low levels of these fungicides as part of an integrated management practice without affecting antagonist viability. ApB was sensitive to the commercial dose of imazalil, but ApB growth was not significantly affected in fruit treated with half of the commercial dose, suggesting that fruit treated with antagonists could be treated with this dose of imazalil, without affecting viability of the antagonist.

ApB was able to grow in a wide range of temperatures, ranging from 1 to 35°C. At 37°C and above, negative growth rates were observed indicating that when exposed to those temperatures viable cells decreased with time. The lack of growth over 35°C is important as a human health safety factor.

Results indicated that chitosan chloride (1%) affected the growth of ApB in apple wounds (Figure 5). However, the use of a combination of the antagonist and chitosan did not appear to affect the biocontrol capacity of the antagonist (Figure 6). When applied separately, chitosan chloride protected 55% of treated wounds while protection with the antagonist alone was about 70%. The combination of both treatments also protected about 70% of treated wounds indicating that there was no additive or synergistic effects when the two agents were applied in combination and that ApB did not benefit from addition of chitosan.

Regarding the mechanism of action of biocontrol agents, lytic enzymes produced by yeasts are believed to play a role in their activity (Castoria et al. 2001; Fan, Tian, Liu, and Xu 2002; Bar-Shimon et al. 2004). The results of this study demonstrated that ApB produced both chitinase and β-1,3-glucanase at 5°C in the presence of *P. expansum* cell walls in both a minimal medium and in apple juice. Production of these enzymes at 25°C, by an *A. pullulans* isolate has previously been reported by Castoria et al. (2001) The present work adds to this information by demonstrating the kinetics of antifungal enzyme production at low temperatures related to yeast growth in the presence and absence of fungal cell walls. At 5°C, a lag phase of approximately 7 and 10 days was observed for glucanase and chitinase production, respectively. This suggests that both enzymes may not play a role in the initial mechanism of action when the antagonist and the pathogen first come into contact within the wound site but rather play a role in long-term protection of the wound site from pathogen infection.

The current study also demonstrated that ApB produced hydroxamate-type molecules, which can act as siderophores, when grown in media containing low concentrations of iron. The production of such compounds may play a role in *Botrytis cinerea* inhibition, as demonstrated in dual cultures in media containing low concentrations of iron (Figure 7). In contrast, *P. expansum* hyphal growth was not affected in dual cultures with the *A. pullulans* strain. This could be explained by a lower sensitivity of *P. expansum* to iron deprivation as demonstrated by Saravanakumar, Ciavorella, Spadaro, Garibaldi, and Gullino (2008).

The role of iron competition as a mechanism in biocontrol of postharvest pathogens in apples and grapes has been previously suggested by Sansone, Rezza, Calvente, Benuzzi, and Sanz de Tosetti (2005), who demonstrated the role of rhodotorulic acid (a siderophore produced by yeasts belonging to the genus *Rhodotorula*) in the control of *Botrytis cinerea*. Additionally, Saravanakumar et al. (2008) demonstrated that iron depletion caused by a selected strain of *Metschnikowia pulcherrima* inhibited the growth of *Botrytis cinerea* and *Alternaria alternata in vitro* and in apple wounds. It was suggested by Sipiczki (2006) that *M. pulcherrima* strains

inhibited the growth of fungal pathogens by immobilizing iron from the medium, forming a red, insoluble pigment called pulcherrimin. The intensity of the color increased when iron concentration increased. By the formation of this insoluble complex, it was suggested that iron remained in the medium but was inaccessible. Siderophores were not involved in this process of tying up iron. In our study, an orange pigment was detected when ApB was grown in dual cultures with *B. cinerea* when the medium contained a high concentration of iron. The nature of that pigment and its relationship to *B. cinerea* inhibition, however, is not known. The current study has provided evidence for an apparent role of iron depletion in *B. cinerea* inhibition by ApB. However, further experiments are needed to determine if the depletion of iron in the surrounding medium by the antagonist is caused by the production of siderophores, by the immobilization of iron in an insoluble pigment, or a combination of both.

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